DTIC
ELECTE
S D
JUL 2 3 1986

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
UNCLASSIFIED	

AD-A170 200

	3. DISTRIBUTION/AVAILABILITY OF REPORT
	Approved for public release; distribution unlimited.

2b. ...ILE / ER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
	AFOSR·TR· 86 - 0 4 9 6

6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
Yale University School of Medicine		Air Force Office of Scientific Research/NL

6c. ADDRESS (City, State and ZIP Code)	7b. ADDRESS (City, State and ZIP Code)
P.O.Box 3333 New Haven, CT 06510	Building 410 Bolling AFB, DC 20332-6448

8a. NAME OF FUNDING/SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
AFOSR	NL	F 49620-82-C-0050

8c. ADDRESS (City, State and ZIP Code)	10. SOURCE OF FUNDING NOS.			
	PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT NO.
Building 410 Bolling AFB DC 20332-6448	61102F	2312	A5	

11. TITLE (Include Security Classification)
Studies of Organophosphate Effects on Retinal Physiology, Cell Biology and Biochemistry

12. PERSONAL AUTHOR(S)
Ted W. Reid and Peter J. Stein

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Yr., Mo., Day)	15. PAGE COUNT
Final	FROM 4/1/82 TO 3/31/84	September 1, 1985	31

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB. GR.	DFP, vision, ERG, growth factor, fluoride, GTPase, phosphodiesterase, DNA synthesis

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

We have approached the problem of DFP effects on the eye in several ways: 1) Dose response curves for DFP inhibition of cellular synthesis of DNA was studied in three different cell types. 2) Electroretinograms (ERG's) were recorded from isolated retinas of Bufo marinus during superfusion with the compound. 3) Intracellular recordings were made from rod photoreceptors in isolated retinas while superfusing with NaF. 4) The effects of fluoride on the protein-protein interactions and the enzymology of the cyclic nucleotide cascade of rod outer segments were studied. 5) DFP binding to rod outer segment and retinal proteins was observed. 6) We have measured the transport of DFP across the cornea.

The data obtained show that both DFP and fluoride alter various enzymatic and physiological functions in cultured cells, the isolated retina, and rod outer segment membranes. The data obtained in the project reveal that both DFP and fluoride may alter ocular biochemistry and physiology through other than classical acetylcholinesterase mechanisms. While a number of the studies performed are preliminary, the data are sufficiently interesting to demand further investigation.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS ☐	UNCLASSIFIED

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE NUMBER (Include Area Code)	22c. OFFICE SYMBOL
Dr. William O. Berry	(202) 767-5021	NL

DTIC FILE COPY

DD FORM 1473, 83 APR   EDITION OF 1 JAN 73 IS OBSOLETE.   UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS P

86 7 23 053

AFOSR·TR· 86-0496

August 1, 1985

FINAL PROGRESS REPORT

AIR FORCE CONTRACT

#F49620-82-C-0050

Ted W. Reid

P. J. Stein

86 7 23 053

DISCLAIMER NOTICE

THIS DOCUMENT IS BEST QUALITY
PRACTICABLE. THE COPY FURNISHED
TO DTIC CONTAINED A SIGNIFICANT
NUMBER OF PAGES WHICH DO NOT
REPRODUCE LEGIBLY.

Introduction:

The organophosphates are an important class of compounds routinely employed as pesticides and chemical weapons. We have investigated the effects of an organophosphate, diisopropylfluorophosphate (DFP) and one of its hydrolysis products, fluoride ion, on various biochemical and physiological parameters in ocular tissues. The data obtained show that both DFP and fluoride alter various enzymatic and physiological functions in cultured cells, the isolated retina, and rod outer segment membranes. While a number of the studies performed are preliminary, the data are sufficiently interesting to demand further investigation. The data obtained in this project reveal that both DFP and fluoride may alter ocular biochemistry and physiology through other than classical acetylcholinesterase mechanisms.

We have approached the problem of DFP effects on the eye in several ways: 1) Dose response curves for DFP inhibition of cellular synthesis of DNA was studied in three different cell types. 2) Electroretinograms (ERG's) were recorded from isolated retinas of _Bufo marinus_ during superfusion with the compound. 3) Intracellular recordings were made from rod photoreceptors in isolated retinas while superfusing with NaF. 4)The effects of fluoride on the protein-protein interactions and the enzymology of the cyclic nucleotide cascade of rod outer segments were studied. 5) DFP binding to rod outer segment and retinal proteins was observed.

In addition we have looked at the transport of DFP across the cornea.

Methods:

Inhibition of DNA synthesis:

For all studies of the inhibition of DNA synthesis we used cells plated in 24 well plates at a level of $1.5 \times 10^{+6}$. After treatment with the inhibitor for various periods of time and at different concentrations, tritiated thymidine would then be added to the cells. After 12 hours the cells were fixed in 5% TCA. They were then washed 5 times and the cell then dissolved in .5M NaOH. This material was then counted in a scintillation counter.

Electroretinograms:

All procedures were performed under dim red illumination or in complete darkness. Bufo marinus were dark adapted 12-18 hours before sacrifice. After sacrifice, the eye was excised from the animal, hemisected and placed in a small Petri dish containing oxygenated Ringer's solution (111 mM NaCl, 2.5 mM KCl, 0.86 mM CaCl , 1.6 mM MgCl , 3.0 mM HEPES, 5.6 mM glucose, and adjusted to pH 7.8 with 1N NaOH). The retina was dissected away from the pigment epithelium and placed receptor side up on a piece of filter paper. The retina was mounted in a sloped perfusion chamber. The recording electrode was a glass pipette filled with Ringer's and agar, that was mounted on a micromanipulator and placed in contact with the retina. The reference electrode was a loop of chlorided silver wire that was placed on the floor of the perfusion chamber below the filter paper and retina. Signals from both inputs were fed differentially into an AC amplifier and recorded with a Gould 'Brush' Recorder. The retina was stimulated with full field flashes of

duration. The flashes were attenuated with appropriate neutral density filters. The preparation was superfused with either normal Ringer's or 5 mM aspartate Ringer's to block neurotransmission from the photoreceptor to second order cells. After mounting and starting perfusion, the retinas were allowed to equilibrate for 30 minutes in darkness. Test solutions contained 1 or 4 mM DFP.

Intracellular Recordings:

All procedures were performed with dim red light or with infrared image conversion. Animals (Bufo marinus) were dark adapted over night (12-18 hours). After sacrifice, the eyes were excised and the retinas were peeled free of the pigmented epithelium. The retina was mounted and superfused in a small dish on the stage of an inverted microscope. The Ringer's solution was identical to the normal Ringeer's solution described above. The test Ringer's contained 1 or 10 mM NaF. Full field flashes of 500 nm light of 0.1 second duration were presented. Attenuation was accomplished through the use of neutral density filters.

DFP Binding Studies:

Retinas were incubated with tritium labelled DFP (20 uCi/retina; 10 uM DFP) in amphibian Ringer's solution (as above) for 5 hours. After the incubation period, the retina was gently washed with normal Ringer's solution until no detectable radioactivity appeared in the wash. Labelling with H^3-DFP was also performed after a 30 minute preincubation with the hydrolysis resistant acetylcholinesterase inhibitor, bethanecol. Following the wash, rod outer segments were separated from the rest of the retina (as described below). The radioactivity in the ROS fraction and the pellet were determined by liquid scintillation counting. Aliquots of each fraction were solubilized in 3% SDS and analysed on 12% polyacrylamide gels. After staining, the gels were sliced, dissolved in 30% H_2O and then subjected to liquid scintillation counting.

Rod Outer Segment Biochemistry:

Preparation of ROS - Retinas were dissected from dark adapted (12-18 hours) Bufo marinus. The retinas were vortexed in 40% sucrose containing 100 mM Tris (pH 7.3), 1 mM $MgCl_2$, and 5 mM DTT (Buffer A) and overlayered with additional Buffer A. The ROS harvested from the sucrose/buffer interface wre resuspended in Buffer A (4 volumes and pelleted at 40,000 x g). The pellet was resuspended in Buffer A (25 uL per retina) and aliquot removed, dissolved in Emulphogene BC-720 and the rhodopsin absorption spectrum recorded before and after bleaching. The rhodopsin concentration was calculated using 40.6 x 10^3 cm/mole as the molar extinction coefficient.

Release of GTP Binding Protein - We assessed the association of the GTP Binding Protein (GBP) with both bleached and dark adapted ROS by monitoring the appearance of the GBP into solution after incubation of the ROS membranes (25-30 uM rhodopsin; 100 uL reaction volume) with KF or guanyl nucleotide followed by centrifugation. At the end of their incubation time, the membranes were centrifuged at 12,000 x g for 30 minutes. The supernatant solutions were recentrifuged under the same conditions for an additional 30 minutes to remove any traces of ROS membrnes. Aliquots were removed in SDS sample buffer (3% SDS) with 0.1 mM

A-1

DTT.   Samples were heated to 90°C. for 5 minutes and subjected to
electrophoresis on 12.5% or 8-20% SDS gels. Gels or gel photographs were
scanned with a Shimadzu Instruments scanning densitometer and peak areas
were determined with a Shimadzu C1-A Integrator.

Trypsin Proteolysis of GBP - ROS membranes were prepared as above and then
washed 3 times in the dark with Buffer A. The membranes were then split
into 3 aliquots. One aliquot was washed three times in the dark with
Buffer A containing 2.5 mM KF. The remaining 2 aliquots were bleached and
washed three times with GTP and GTPyS respectively. Aliquots of each GBP
preparation containing approximately 10 uG of BGP were incubated with
trypsin (7.0 or 14.0 uG/ml, final concentration). At appropriate times,
the proteolysis was stopped by the addition of excess soybean trypsin
inhibitor.   After the addition of 3% SDS sample buffer, the samples were
analysed on 12.5 or 14% polyacrylamide gels.

Phosphodiesterase Activity - To assess the effects of KF washing on
membrane bound phosphodiesterase (PDE) activity, ROS membranes (prepared
as above) were washed 4 times in the dark with Buffer A containing 2.5 mM
KF or KCl. The membranes were diluted to a final rhodopsin concentration
of 1-10 uM (400 uL reaction volume) and assayed in the light for cyclic
GMP hydrolysis.

    To determine the effect of KF incubation on PDE activity, ROS membranes
were preincubated (in the dark) or bleached and preincubated in the light
for 4 minutes with KF or GTPyS in 125 mM KCl, 1 mM MgCl, 5 mM DTT and 2.5
mM Tris (pH 8.0). At the end of the preincubation period, 5 mM cyclic GMP
was added and the rate of proton evolution was monitored using a pH
electrode whose output was fed into a voltage follower and amplifier and
recorded on a Model 220 Brush Recorder.

    Corneal penetration study:

To determine to rate and amount of penetration of the cornea, Rabbit
corneas were mounted in a chamber so that it was possible to moniter the
amount of radioactive DFP that was transported through the cornea with
time. The corneas were in a standard rabbit Ringers solution. The design
of the chamber was such that only the cornea was exposed to the solution.

Results:

    DNA synthesis inhibition by DFP:

    A dose response curve for diisopropylfluorophosphate (DFP) inhibition
of cellular synthesis of DNA was studied in three different cell types.
As seen in figures 1 and 2, the dose response for DFP in the presence of
5% fetal calf serum is the same for Na and mouse lens epithelial cells or
transformed retina cells growing in tissue culture. DFP showed half
maximal inhibition at 4.0 mM for the transformed retina and 2.0 mM for the
lens cells.   In studies with 3T3 fibroblasts, 75% inhibition occured at
3.3 mM DFP (see Figure 3A).   The most dramatic inhibition, however,
occurred when the anticote 2-PAM was added to the culture medium with
DFP.   Figure 4 shows that concentrations of 2-PAM as low as 0.04 mM caused
an increase in the inhibitory effect of DFP. This contrasted strongly
with the stimulatory effects on DNA synthesis observed (Figure 4) when

2-PAM was added without DFP at 0.4 and 0.04 mM. With 3T3 cells, however, a very different result was obtained (Figure 3B). 2-PAM added alone showed no stimulation and was inhibitory at 4 mM (it was not inhibitory with lens epithelial cells at 4 mM). In addition, concentrations of 2-PAM as low as 0.004 mM increased the inhibition of DFP on DNA synthesis.

We then determined if the inhibition by DFP was a result of the action of fluoride generated by DFP hydrolysis. The dose response curve for fluoride (Figure 5) showed that the inhibitory effect was half maximum at about 0.8 mM for cells stimulated with fetal calf serum or retinoblastoma derived growth factor (RDGF). This data suggested that the release of fluoride by DFP might be the cause of the inhibitory effect on DNA synthesis. With this in mind, we preincubated DFP with 2-PAM before adding it into the cells (Figure 6). This was done because we suspected that 2-PAM might have reacting with the DFP causing the release of free fluoride ions. It appears from the results in Figure 6 that preincubation has a small effect. The preincubation time for this experiment was 3 hours and the concentration DFP and 2-PAM was ten times that found in the media over the cells. From the results it is apparent that the reaction time between DFP and 2-PAM must be slow relative to the time for initiation of DNA synthesis, for 2-PAM to have an effect. For this to work, fluoride must be able to inhibit later in the cell cycle. As seen in Figure 7, this appears to be possible because fluoride (alone) has an effect after 19 hours, while there is little difference between DFP alone or added with 2-PAM after 19 hours. However, Figure 7 also shows that there is an inhibitory effect of DFP after only one hour of addition, 19 hours after the initiation of DNA synthesis. To check this effect, DFP was added by itself at different periods of time after the start of the cell through the cell cycle. It can be seen in Figure 8 that there is a rapid effect of DFP that occurs after 1 hour but remains the same for 6 hours. If DFP is present for a longer time, an additional inhibition occurs. These results are consistent with the quick effect resulting from DFP by itself, and the longer time effect resulting from fluoride released by the hydrolysis of DFP.

Electroretinograms:

Superfusion of the isolated toad retina with Ringer's solution containing DFP (1 or 4 mM) results in striking changes in the a and b-waves of the ERG. Figure 9 shows intensity response curves of the a-wave, made before, during, and after the application of DFP. There is a noticeable striking decline in the amplitude of the responses at all stimulus intensities after exposure to DFP. Figure 10 shows the kinetics of the decline in response amplitude to a constant intensity stimulus in Ringer's solution containing 5 mM Na aspartate (to block responses from second order cells). Thus, it appears that DFP alters the physiology of the receptors. Figure 11 shows the changes in b-wave intensity response functions after exposure to DFP. There is an initial increase in amplitude followed by a subsequent decline.

Intracellular Recording:

Intracellular recordings of transmembrane voltage were made from rods of retinas superfused with Ringer's solution containing either 1 or 10 mM NaF. At 1 mM NaF negligible alterations in the membrane potential and no

changes were observed in light induced responses. At 10 mM NaF, the dark
membrane potential was little changed but decreases in response amplitude
could be observed (Figure 12).

DFP Binding:

Figure 13 show the radioactivity profile for ROS incubated with H -DFP
with and without princubation with bethanecol. Three major peaks are
apparent with approximate molecular weights of 180, 31, and 27
kilodaltons. The 180 kilodalton peak disappeared in the bethanecol
pretreated ROS. Minor peaks with approximate molecular weights of 83, 50,
21, and 16 kilodaltons also can be observed. Of the labelled proteins.
only the 50 kilodalton protein corresponds in molecular weight to proteins
previously identified functionally. An ATP binding protein of molecular
weight 48 kilodaltons(1) and a protein kinase of molecular weight 50-53
kilodaltons(2) have already been described.

The retina fraction (pellet) (Figure 14) showed major peaks at 47, 32,
and 29 kilodaltons and minor peaks at 79, 63 and 23 kilodaltons.
Bethanecol did not alter the labelling of any of the proteins in the
retina fraction. The retina fraction inevitably retains some of the outer
segments, hence labelling of proteins of similar molecular weight probably
is indicative of this small contamination (27-29, 31-32, 49-50 kilodalton
peaks).

Rod Outer Segment Biochemistry:

The effects of KF on the association of the GBP with ROS membranes are
shown in Figure 15A and 15B. Figure 15A demonstrates that incubation of
dark adapted ROS membranes with buffer containing KF at various
concentrations followed by centrifugation, results in a concentration
dependent release(lanes b-g) of GBP. By contrast, buffer alone, 10 uM
Gpp(NH)p and 10 uM GTP (lanes a, h, and i) are relatively ineffective in
releasing GBP from the dark membrane. When bleached membranes are
incubated with fluoride, the results are strikingly different. Figure 7B
shows that incubation of bleached ROS membranes with KF followed by
centrifugation. releases only small amounts of GBP into the supernatant
solution (lanes b-g) compared with Gpp(NH)p or GTP (lanes h and i). The
amounts of GBP released under these experiental conditions are illustrated
in Figure 16. Incubation of bleached membranes with fluoride releases, at
most. only 14% of the GBP that can be released by Gpp(NH)p. Incubation of
dark adapted ROS membranes with fluoride release almost 50% of the amount
of GBP released by Gpp(NH)p in bleached membranes. It appears that half
maximal release occurs at 1.0 mM fluoride.

Several previous studies (3,4) showed that the active (capable of
activating PDE) and inactive conformations of GBP may be distinguished by
their digestion patterns during limited trypsin proteolysis. We employed
this technique to analyse the conformation of GBP released with KF.
Figure 17 shows that trypsin digestion of KF-released protein is similar
to that generated for the protein with GTPvS bound. After release from
the membrane with either KF or GTPvS, trypsin digestion generates a 32
kilodalton fragment stable to further digestion. By contrast, when GBP is
extracted with GTP and then subjected to trypsin proteolysis, the
digestion proceeds past the 32 kilodalton stage to generate fragments of
23 and 12 kilodaltons (data not shown). Thus limited trypsin proteolysis

indicates that the conformation of GBP when released by KF in the dark is similar to the 'active' conformation obtained with hydrolysis resistant GTP analogs.

To assess the influence of fluoride on PDE activity, we measured cGMP hydrolysis in dark adapted ROS membranes exposed to buffers containing different KF concentrations. Figure 18 shows that PDE activity is optimally stimulated, in the dark at 5.0 mM KF. Half maximal stimulation appears at about 1.0 mM KF. It is interesting to note that at 10.0 mM KF, enzyme activity is reduced. Inhibition of the catalytic moiety by fluoride may be responsible for this effect (R. Sorbi, personal communication). Similarly adenylate cyclase from rat cerebral cortex is maximally stimulated at 18 mM KF, while concentrations above 25 mM are inhibitory (5).

To analyse the mechanism by which PDE is activated by fluoride, we washed dark adapted ROS membranes with Buffer A containing either 2.5 mM KC1 or KF. The activity of PDE which (unlike GBP) remains membrane bound was then assayed in the light. Table I shows that KCl washed ROS membranes (which contain GBP) require the addition of GTPyS to obtain maximal hydrolytic activity. By contrast, KF washed ROS (depleted of GBP) are maximally active without addition of GTPyS. PDE activity in KF washed ROS membranes is not altered by the addition of GTPyS (data not shown). PDE activity in KF washed ROS membranes was 95-100% of the activity measured in KCL washed membranes in the presence of light and GTPyS. Washing dark ROS membranes with KF appears to remove an inhibitory constraint normally present on the catalytic moiety of PDE. Reconstitution experiments (R. Sorbi, personal communication) support the idea that this restraint is due to the PDE inhibitor molecule.

Penetration studies:

As can be seen in figure 19, the first apperance of DFP on the other side of the cornea is at around 10 minutes. For this experiment no cold DFP was added to the system. In figure 21 two different concentrations of cold DFP was added to teh system. As can be seen when cold DFP is added the DFP still penetrates in about the same time (8-10 min) however there is a slight decrease in the overall rate at higher concentrations of cold DFP.

Discussion:

Inhibition of DNA synthesis:

It was determined that the main effect of DFP as an inhibitor of DNA synthesis was due to the effect of fluoride released from the DFP during its hydrolysis. This was seen in the results that fluoride could mimic the effects of DFP and that 2-PAM would stimulate the inhibitory effect. Thus 2-PAM in its reaction with DFP probably displaces fluoride from the DFP to form a 2-PAM-DFP compound similar to that which results from the reactivation of acetylcholinesterase. It was important to note however that DFP by itself could react and inhibit the reaction in 1 hour which was too short a time for the hydrolysis to become appreciable as seen from the fact that incubation with 2-PAM had no effect on this early reaction. Thus these results are consistent with the quick effect resulting from DFP by itself, and the longer effect resulting from flouride released by the

hydrolysis of DFP.

Electrophysiology:

Extracellular recording from the amphibian retina in the presence of DFP resulted in a monotonic decrease in the amplitude of the a-wave. This effect is surprising since there is no evidence for the presence of acetylcholinesterase in this cell type. The data would indicate that it is possible that this class of compounds may exert deleterious effects through other than the classical pathway. Exposure to DFP under the conditions employed resulted in irreversible decreases in the a-wave response. More extensive testing of different DFP concentrations and exposure periods are necessary to better understand the nature of the interaction between DFP and the rod photoreceptor. The time course of the decay of response amplitudes of the retina must be better characterized under normal recording conditions.

Intracellular recordings from rod photoreceptors of retinas superfused with KF indicate that the effects of DFP on the extracellularly recorded a-wave do not result from the generation of fluoride ions by DFP hydrolysis. No changes were observed in intracellular responses from the rods were observed when the retina was superfused with 1 mM KF. It is unlikely that more than uM concentrations of fluoride ions could be generated under these experimental conditions. By contrast, changes in the amplitude of the rod responses were observed with 10 mM KF in the superfusate. Additional experiments are clearly needed at additional KF concentrations and exposure times to better characterize the effects of fluoride and to begin to understand the cellular mechanisms which this ion may affect. It would also be useful to attempt to record intracellularly with and without superfusion with DFP.

DFP Binding:

DFP binding studies indicate that there are proteins in both the outer segment and the retina which can bind this molecule. The identity and functional properties of these proteins remain to be elucidated. Further studies should help to define the mechanisms by which DFP exerts its effects on retinal physiology. Since DFP can label proteins in the retina, autoradiographic techniques to localize the DFP binding sites will be useful in identifying the cell types affected by this molecule. A comparison of DFP localization with histochemical localization of acetylcholinesterase should provide additional evidence that DFP can alter metabolism by acting through non-acetylcholinesterase pathways. Additional studies of the stoichiometry of DFP binding to specific proteins would be useful as well.

Rod Outer Segment Biochemistry:

Incubation of dark adapted ROS membranes with KF containing buffer results in a change in the conformation of the GBP such that it is capable of activating PDE. This change is also indicated by the KF concentration-dependent release of the protein from the ROS membrane and the fact that the trypsin digestion pattern resembles that of the GBP with hydrolysis resistant analog bound. Since, in this state, the protein is in a conformation capable of activating PDE, it appears that fluoride

activation of PDE is mediated by the GBP. This idea is supported by reconstitution experiments which show that the fluoride solubilized GBP can be added back to dark adapted ROS membranes and activate the enzyme (R. Sorbi, personal communication.

Both fluoride and guanine nucleotides release the GBP from ROS membranes. However, fluoride releases the protein most efficiently in dark ROS membranes while guanine nucleotides are most efficient in bleached membranes. Washing dark adapted ROS membranes after exposure to fluoride activates PDE. The mechanism of activation appears to involve release of an inhibitory protein from the catalytic moiety (R. Sorbi, personal communication). A similar mechanism was recently demonstrated to account for light dependent guanyl nucleotide activation of PDE (6). However, fluoride activation occurs in dark adapted membranes while guanyl nucleotide activation requires bleaching. Thus, the light dependent rhodopsin/GBP interaction required for guanyl nucleotide activation of PDE is apparently not required for fluoride activation of this enzyme. This parallels observations made for adenylate cyclase where hormone (and hormone receptor) is not necessary for fluoride activation of the enzyme (5,7). Furthermore, fluoride activation of the adenylate cyclase stimulatory GTP binding protein (N) has been shown to resemble activation by GTP analogs (8). The trypsin digestion experiments reported above demonstrate that the GBP released by fluoride or GTPyS generate identical proteolytic fragments and therefore have similar conformations. Since photoreceptor GBP can substitute for adenylate cyclase GBP in the activation of the catalytic moiety (9), it appears likely that a similar conformation change must occur in the adenylate cyclase GBP during the activation process.

Penetration studies:

The results from the cornea penetration studies are consistent with DFP penetrating through the cornea in about 8-10 minutes. Also when cold DFP was added to the system in high concentrations, the fact that the rate was slightly smaller seemed to imply that DFP was being facilitated in its transport through the cornea. Whether or not there is a specific transport protein will require further detailed studies.

References

1. Zuckerman, R., Budzygon, B. and Liebman, P.A. (1984) Invest. Opthal. and Visual Sci. 25:112.

2. Shichi, H. and Somers, R.L. (1978) J. Biol. Chem. 253:7040-7046.

3. Fung, B.K.-K., and Nash, C. (1983) J. Biol. Chem. 258:10503-10510 .

4. Halliday, K.R., Stein, P.J., Chernoff, N., and Bitensky, M.W. (1984) J. Biol. Chem. 259:515-525.

5. Rasenick, M.M. and Bitensky, M.W. (1980) Proc. Natl. Acad. Sci. U.S.A. 77:4628-4632.

6. Yamazaki, A., Stein, P.J., Chernoff, N., and Bitensky, M.W. (1983) J. Biol. Chem. 258:8188-8194..

7. Downs, R.W., Spiegel, A.M., Singer, M., Reen, S., and Aurbach, G.D. (1980) J. Biol. Chem. 255:949-954.

8. Sternweis, P., Northup, J.K., Smigel, M. and Gilman, A.G. (1981) J. Biol. Chem. 256, 11517-11526.

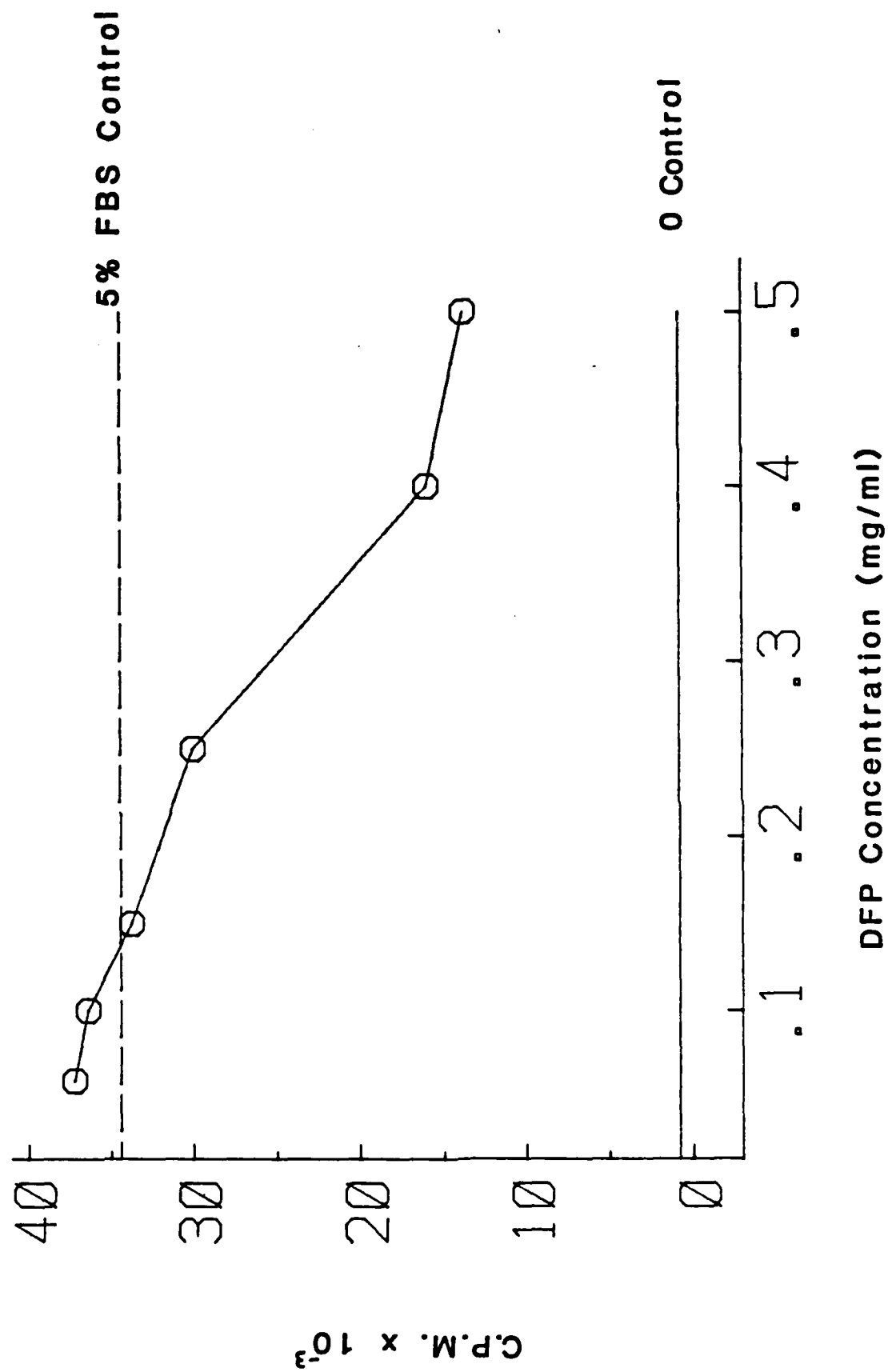9. Rasenick, M.M., Stein, P.J., and Bitensky, M.W. (1981) Nature (London) 294:560-562.

Figure 1

C.P.M. x 10^3

DFP + 5% FBS   (HI 90 min)

C.P.M. x 10^3

DFP + 5% Nu -Serum

Log DFP Concentration (mg/ml)

Log DFP Concentration (mg/ml)

C.P.M. $\times$ 10^{-3}

(1.9 mM)

(2.7 mM)

(4.3 mM)

(6.8 mM)

2-PAM(3.0 mM)

Figure 3A

5% FBS Control

0 Control

C.P.M. x 10^{-3}
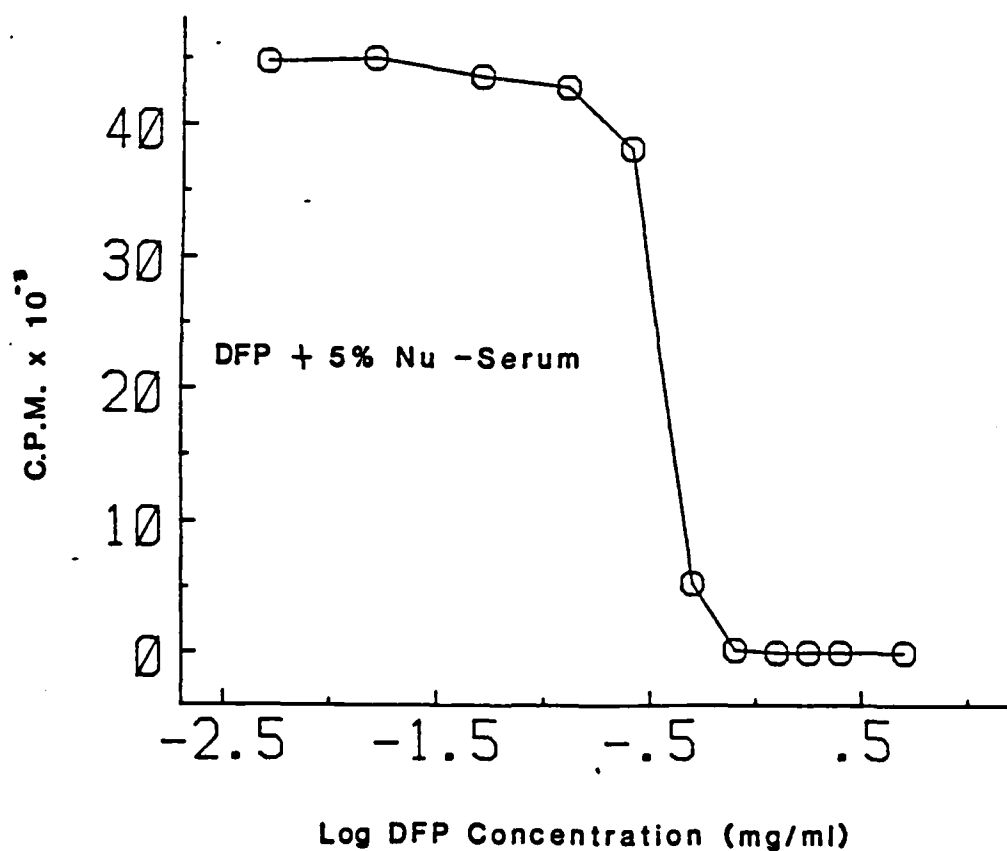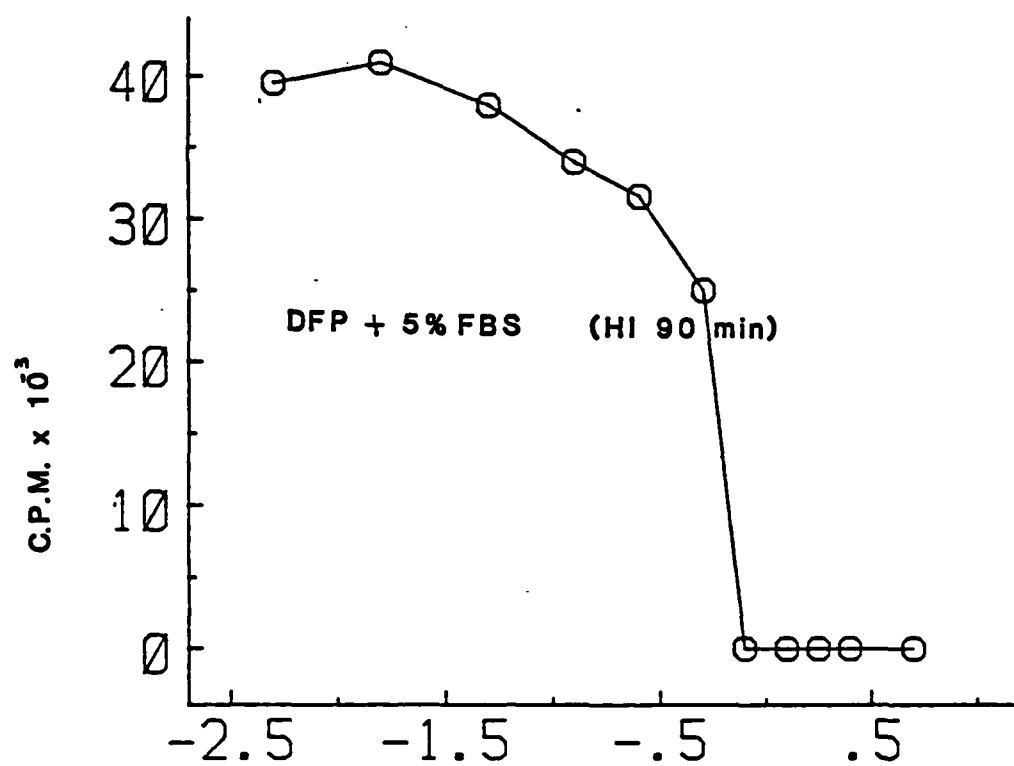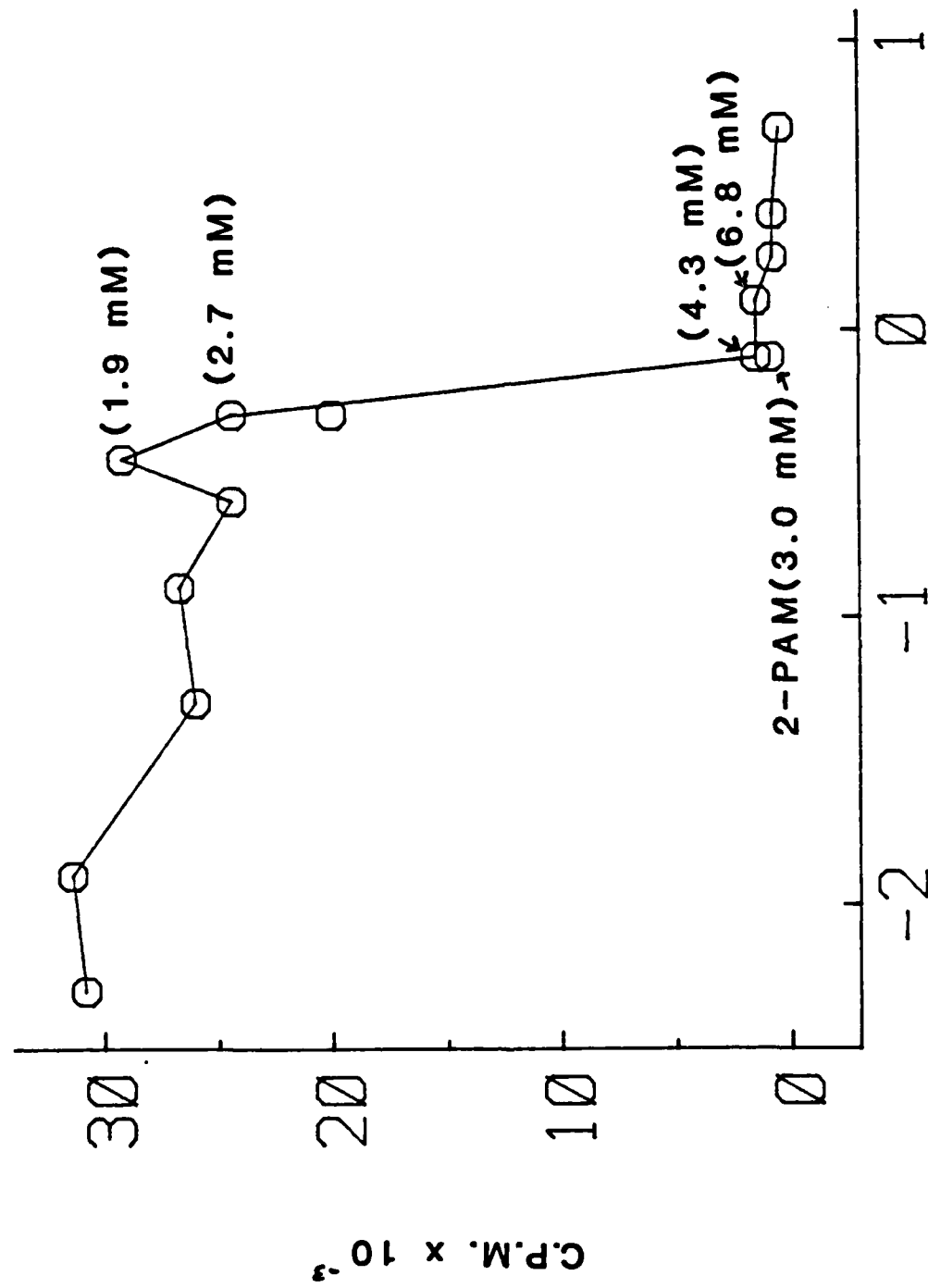
DFP Concentration (mg/ml)

Figure 38

CPM x 10⁻³
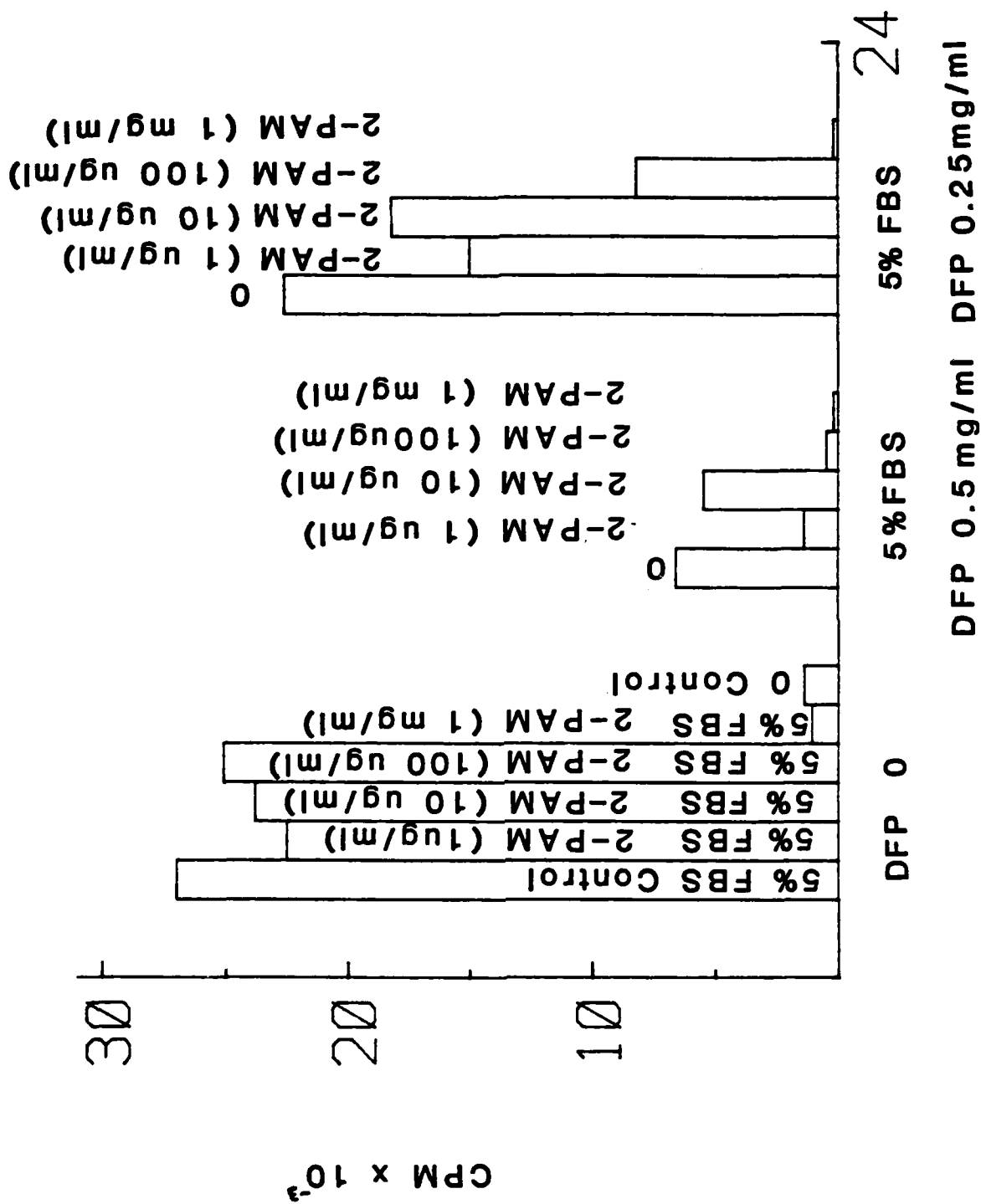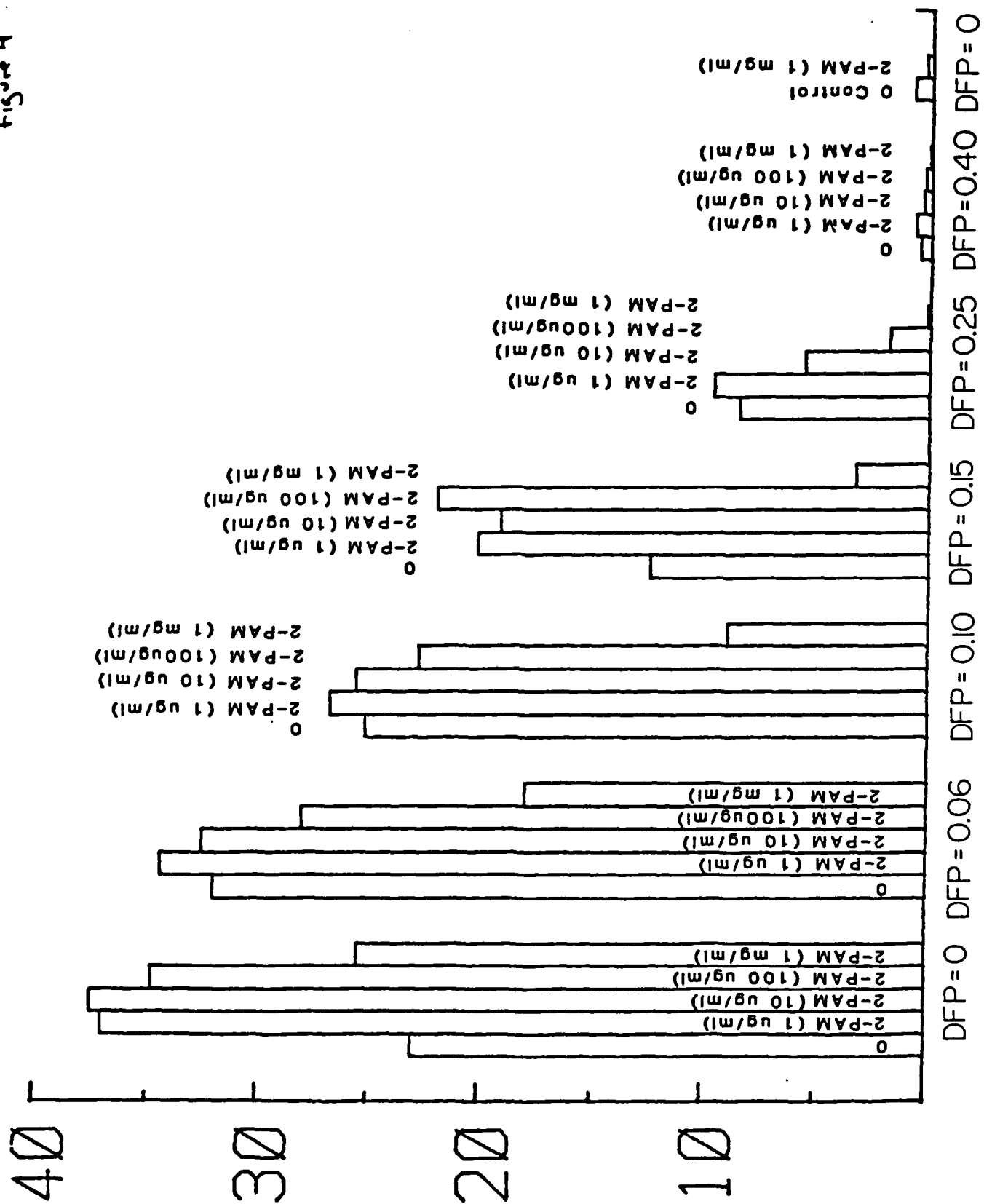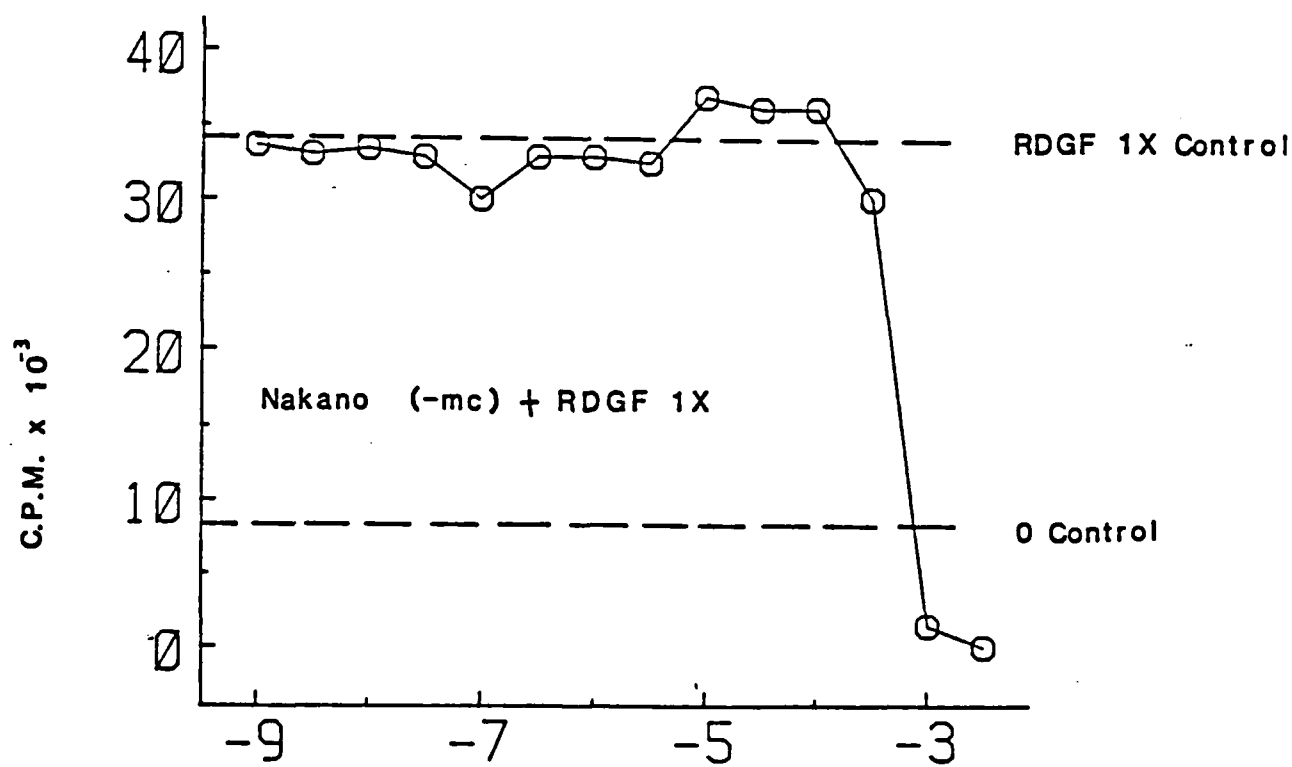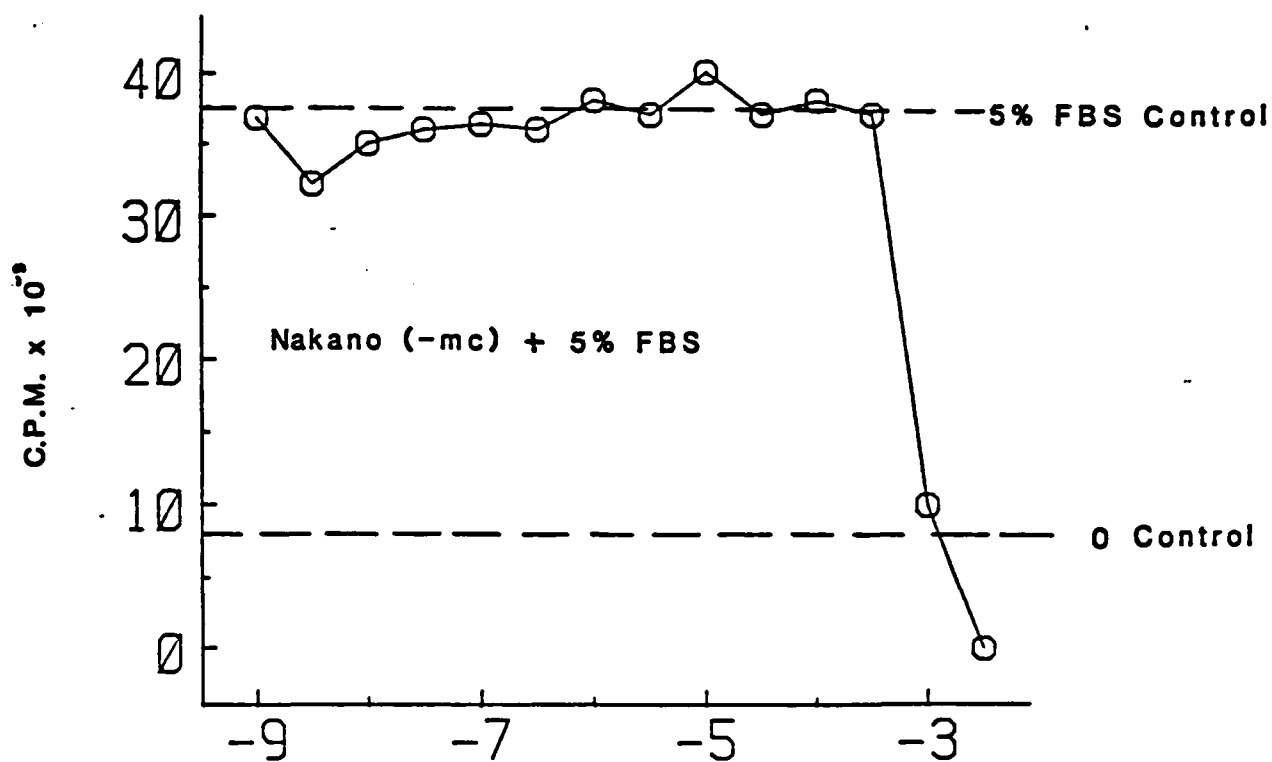
Figure 4
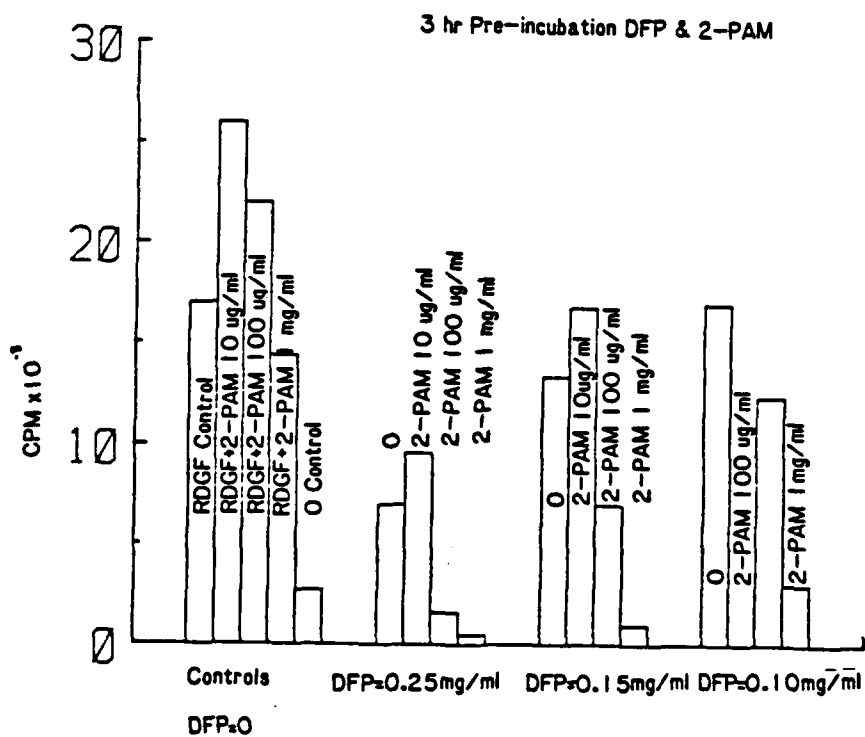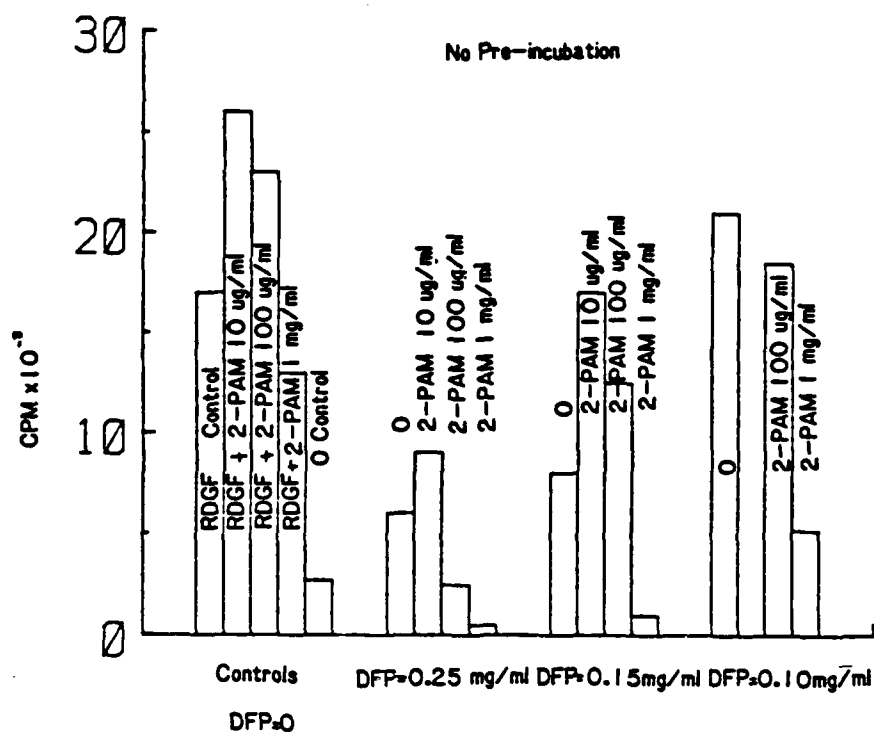
Figure 5

Figure 6

Figure 7

DFP = 0.25 mg/ml
2-PAM = 0.36 mg/ml
NaF = 0.059 mg/ml

No Washout of RDGF

1 hr Washout of RDGF
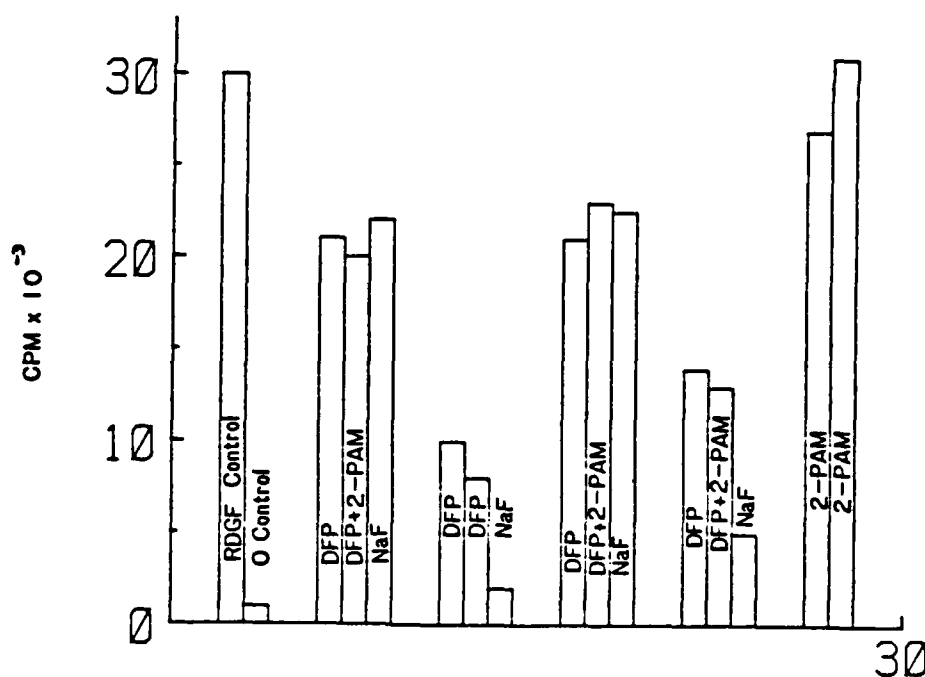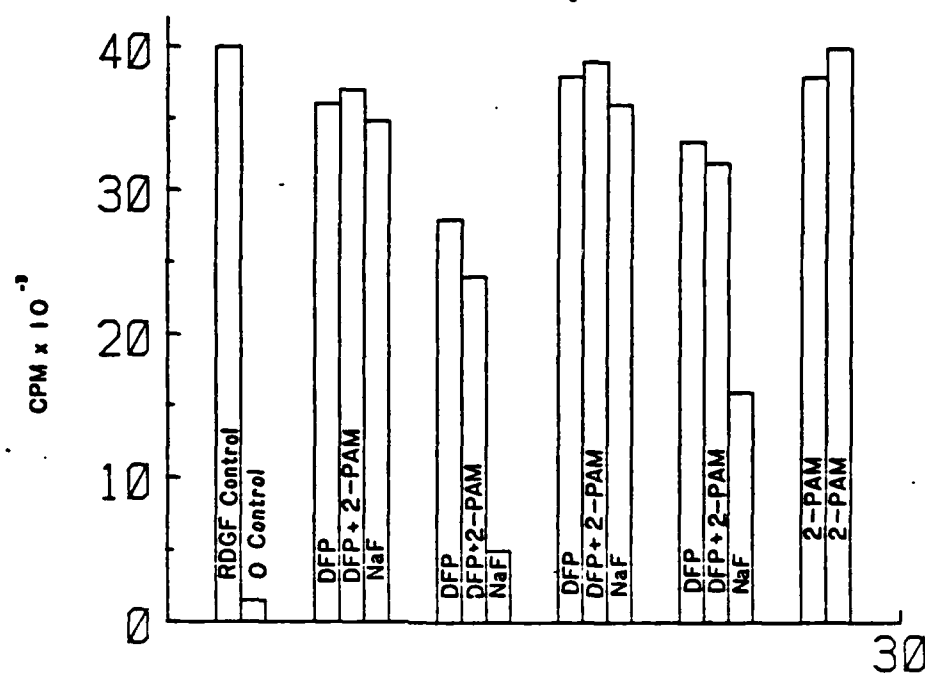
Figure 8

figure 7.

figure 10

Amplitude of a-wave (uV)

5.0 mM Aspartate – Ringer's

Time in Minutes
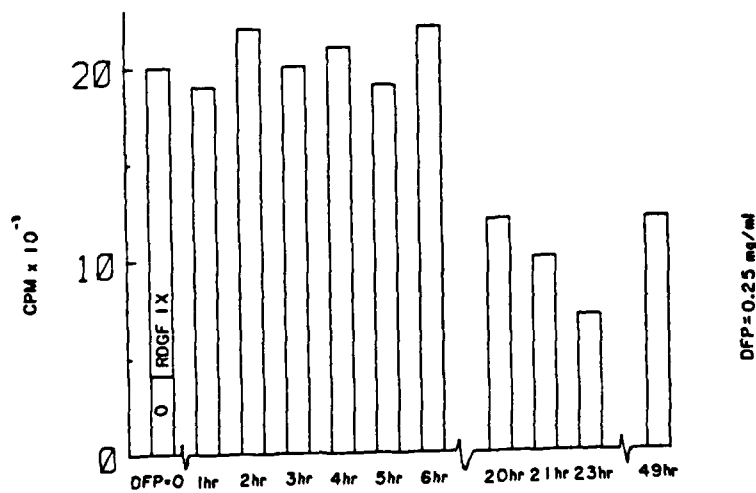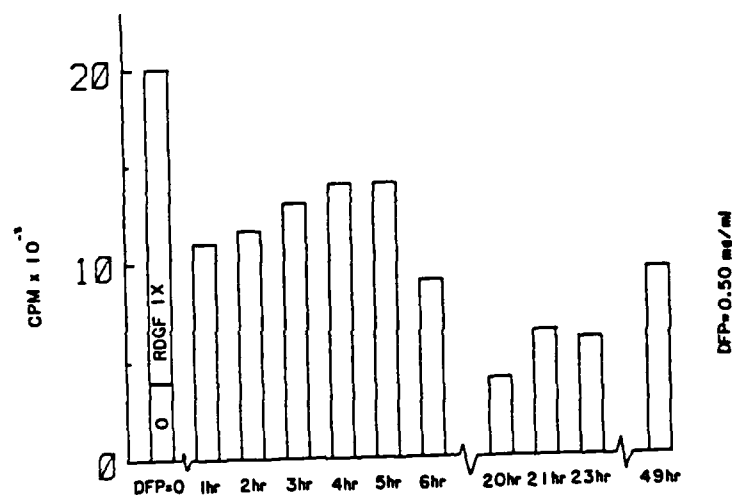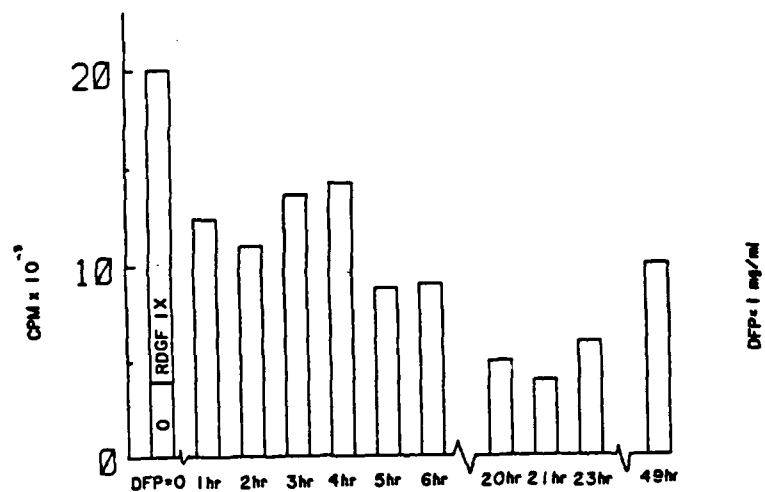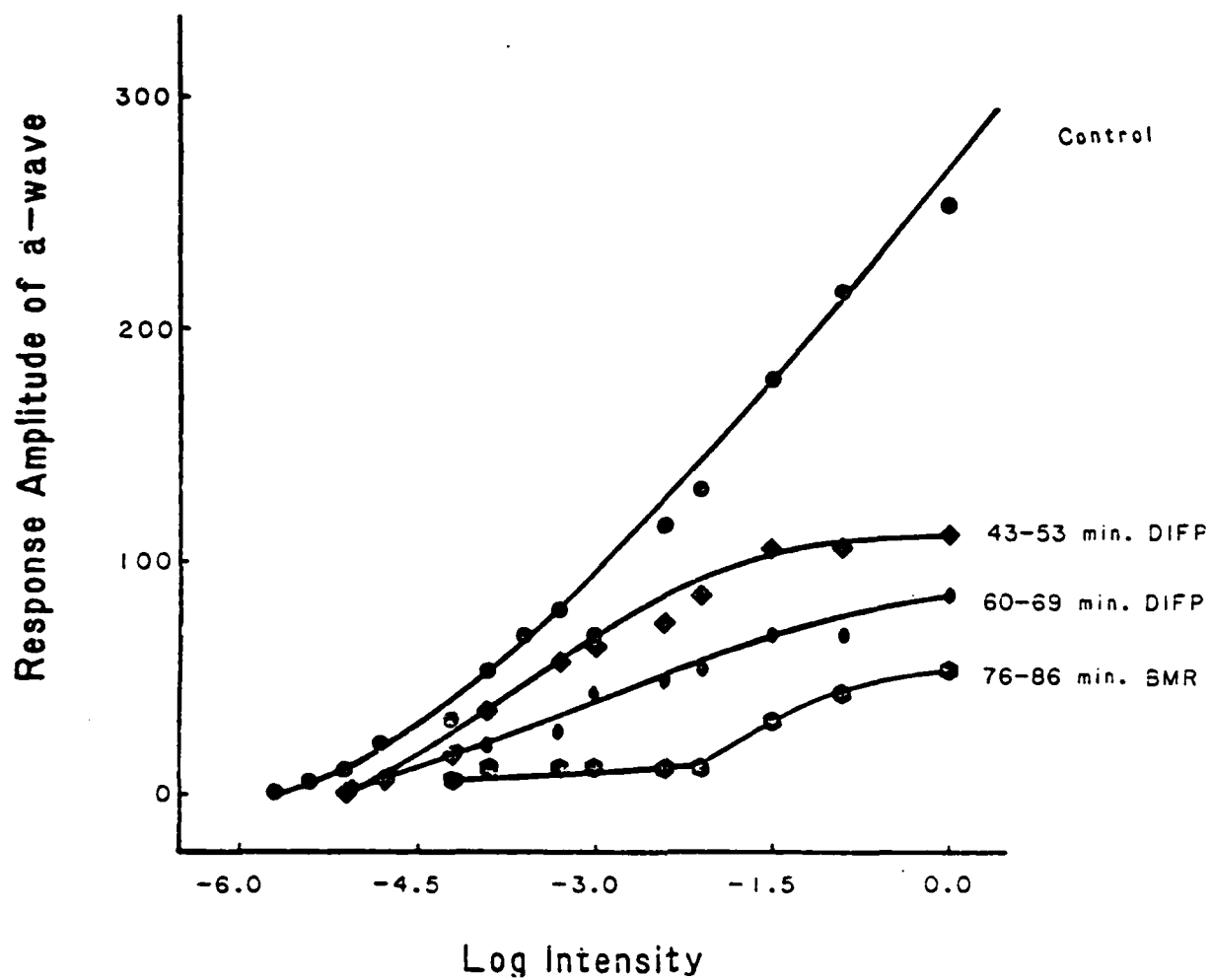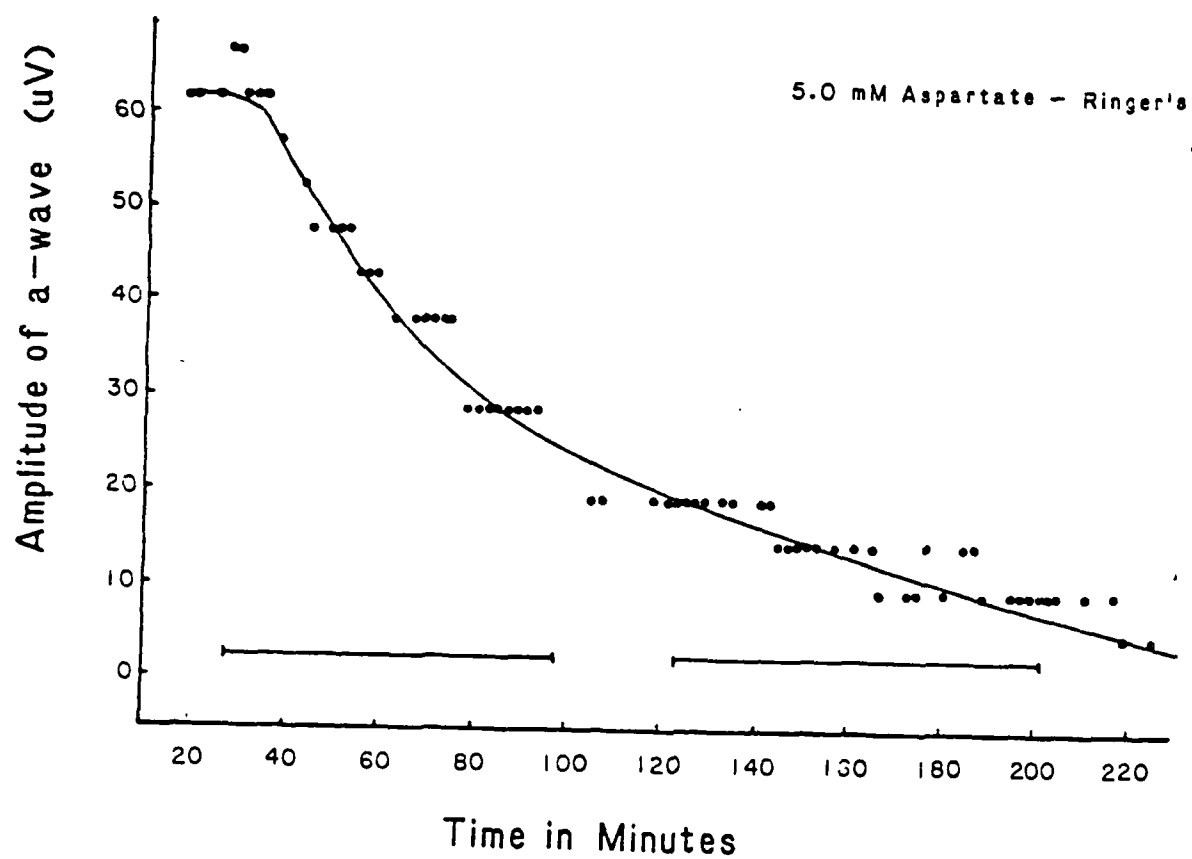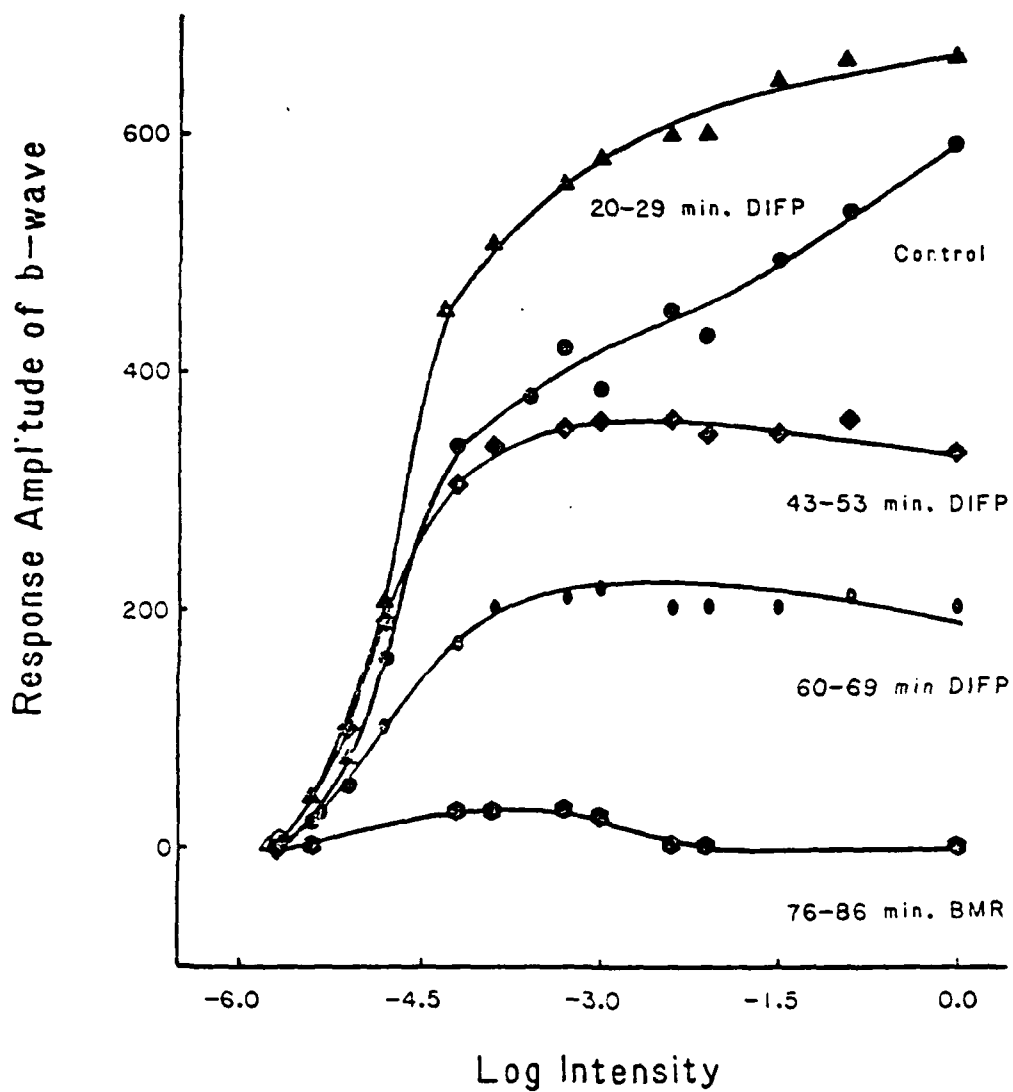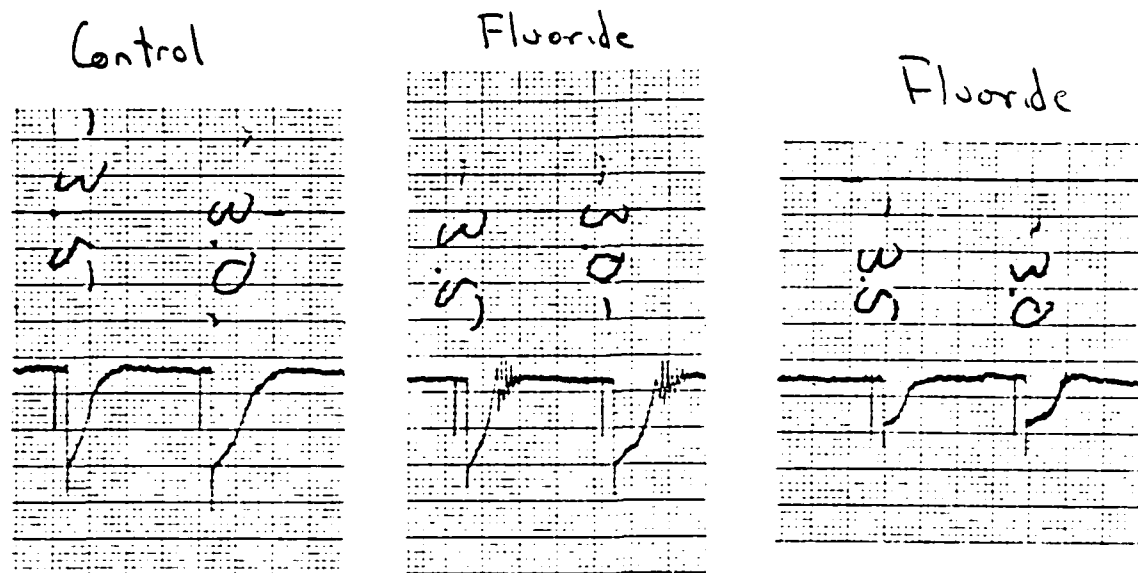
Figure 11

Figure #12

Light evoked responses to two different intensity flashes before and after exposure to 10 mM f NaF. Calibration pulse before hyperpolarization is 10 mV.
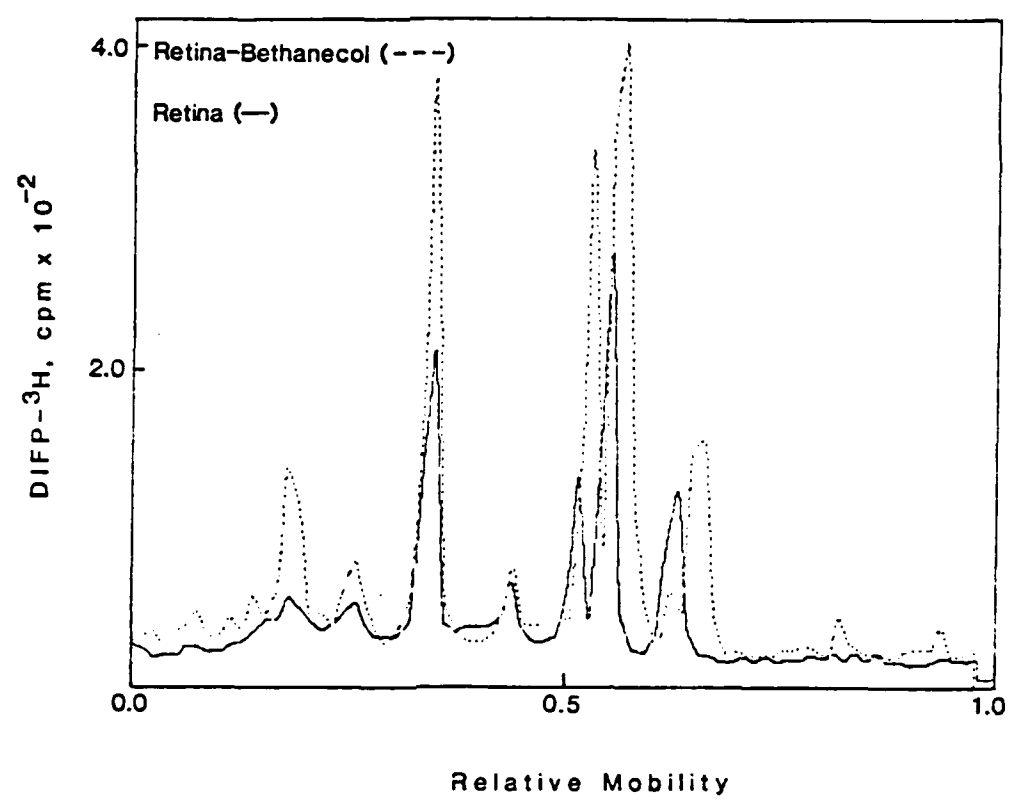
Figure 213

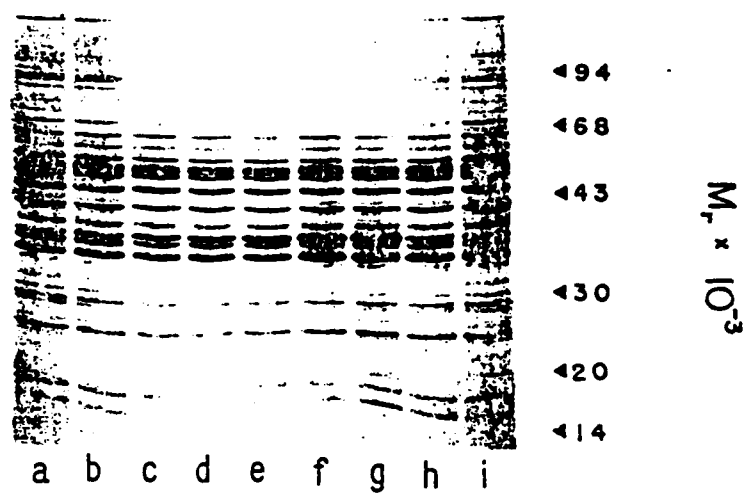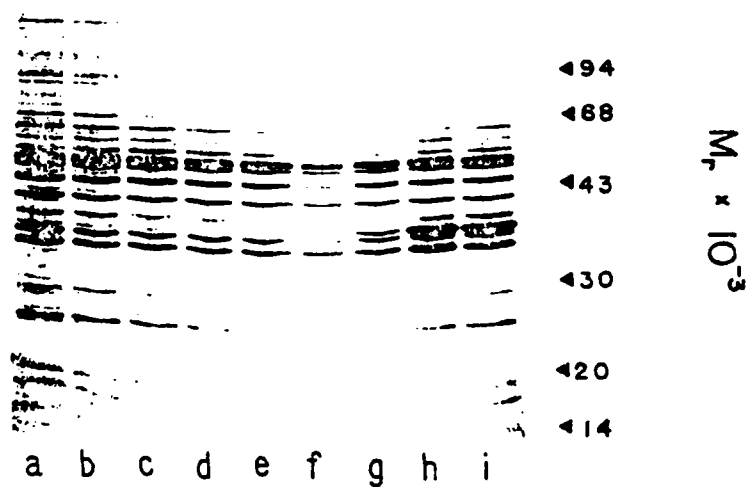ROS-Bethanecol (-----)

ROS (———)

DIFP-³H, cpm x 10⁻²

Relative Mobility

Figure 6 /4

Figure showing graph with y-axis "DIFP-^{3}H, cpm $\times$ 10^{-2}" ranging from values with markers at 2.0 and 4.0, and x-axis "Relative Mobility" from 0.0 to 1.0. Legend: Retina-Bethanecol (– – –), Retina (—).

Figure V 15

A. Dark

B. Light

figure 8.16

X-axis: Fluoride Concentration (mM)

Y-axis: % GBP Released

$$M_r \times 10^{-3}$$

G FLUORIDE   G GTP-ɣ-S

39
36
32
26

15

a b c d e a' b' c' d' e'

Figure 10

% Light Activation (y-axis)

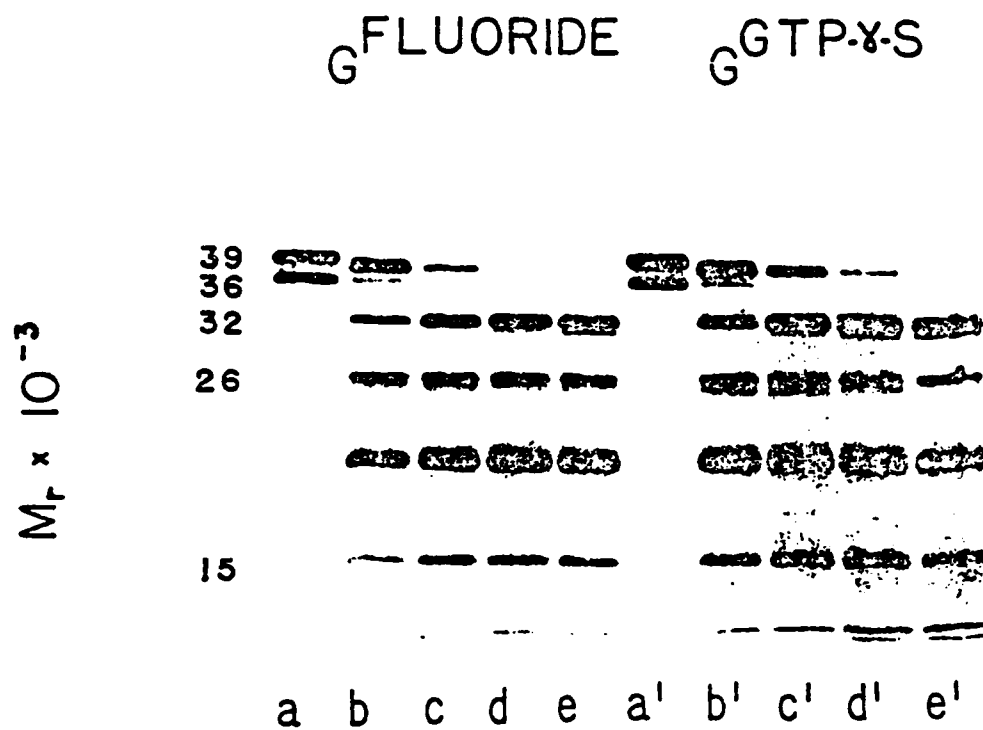**Fluoride Concentration (mM)** (x-axis)
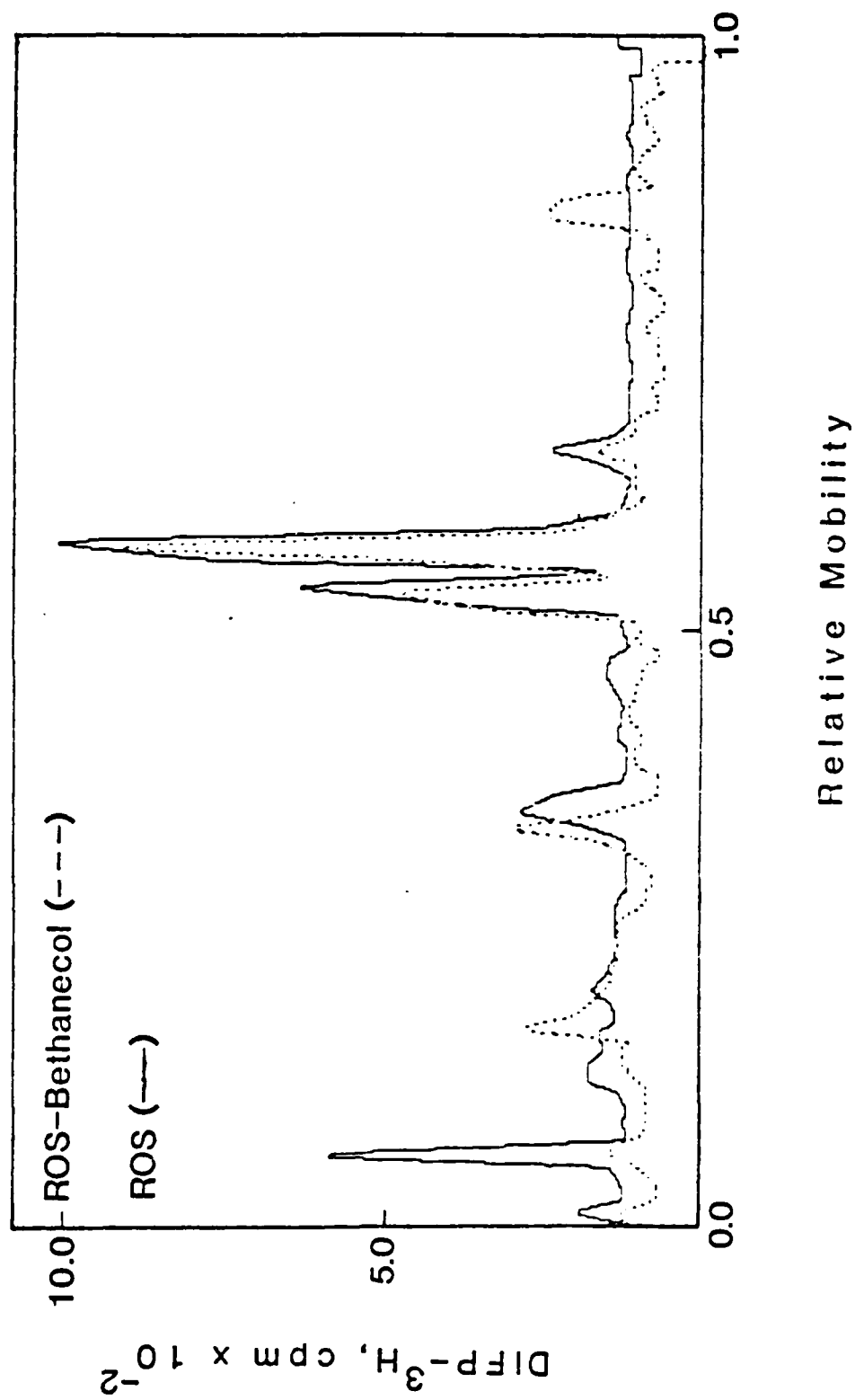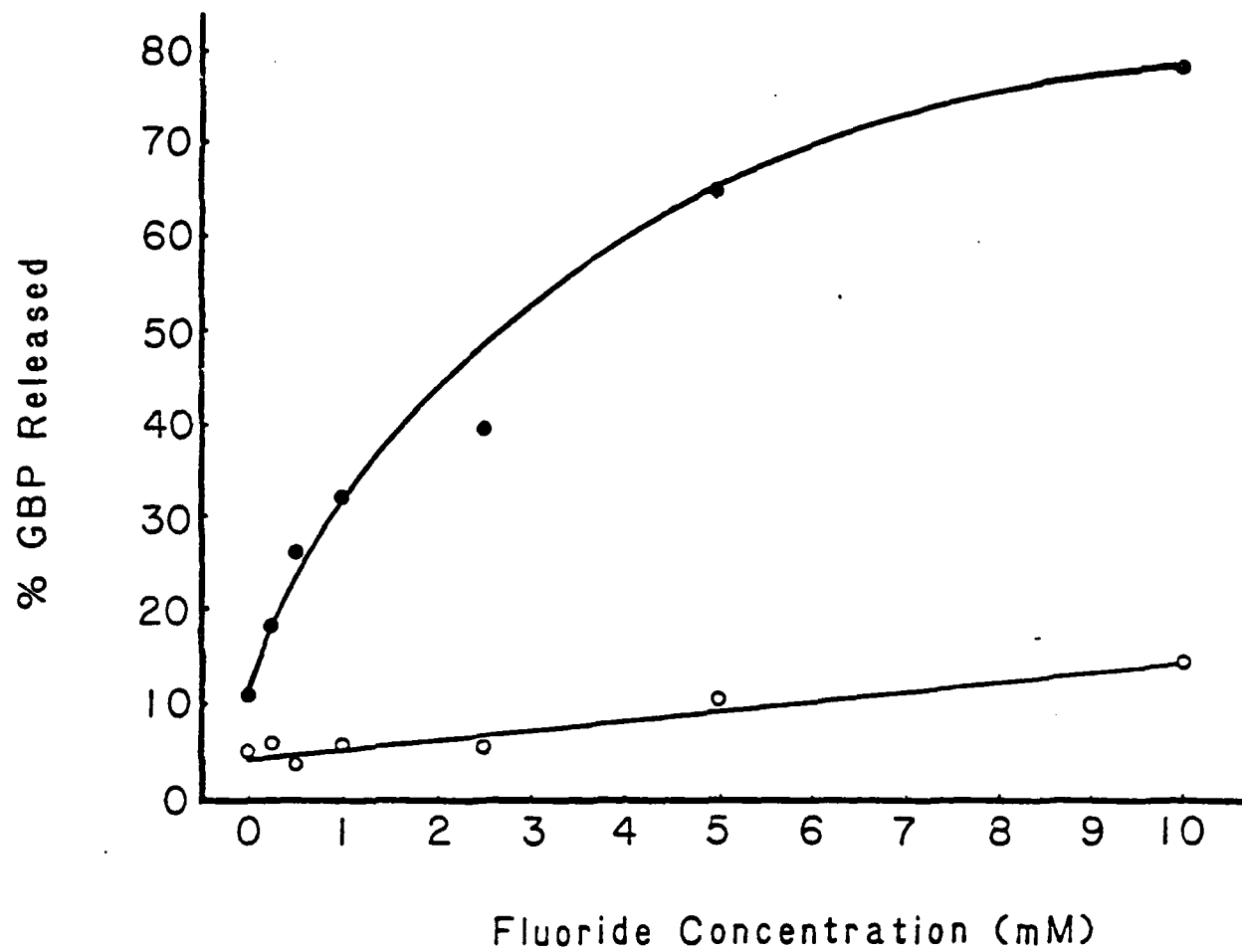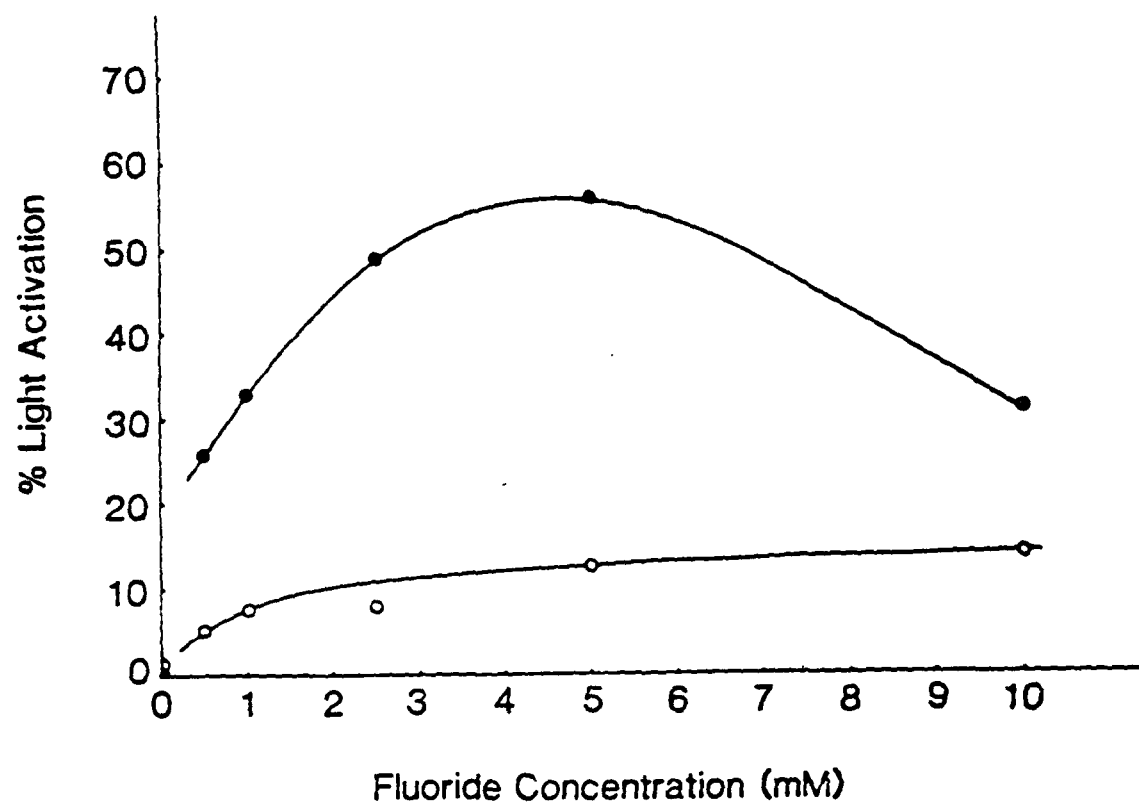
Table I

PDE Activity of KCl and KF Washed ROS Membranes

	Additions:	Activity (M cGMP/M Rhodopsin min)
KCl Washed ROS	None	24
KCl Washed ROS	GTFYS	355
KF Washed ROS	None	350

Figure 19

Figure 20

Cornea Penetration
(+ Unlabelled DFP)

END

DTIC

9-86